REMARKS

Claim Amendments:

The claims have been amended to simplify the invention and to more particularly describe the invention. First, Claim 1 has been amended to limit the claims to CGRP, biologically active fragments thereof and biologically active homologues thereof. Support for this amendment and the amendment to Claims 40 and 41 is found in the specification on page 25, line 23 to page 26, line 23.

Supplemental Information Disclosure Statement filed April 23, 2003 and Declaration Under 37 CFR 1.131:

On April 23, 2003, Applicants filed a Supplemental Information Disclosure Statement. However, since the Examiner mailed the final Office Action on April 22, 2003, the documents crossed paths in the mail and the reference submitted has not yet been considered on the record. In conjunction with the filing of the present Request for Continued Examination, Applicants respectfully request that the Examiner consider U.S. Publication No. 2002/0037846A1. In addition, Applicants provide the following comments on the reference.

Applicants submit that U.S. Publication No. 2002/0037846A1 is not effective prior art against the claimed invention because the claimed subject matter was invented by the present inventors prior to the effective date of U.S. Publication No. 2002/0037846A1 (December 30, 1999) and prior to the earliest priority date of U.S. Publication No. 2002/0037846A1 (December 24, 1999). Enclosed herewith is a Declaration under 37 CFR § 1.131 executed by both of the present inventors. This Declaration provides evidence of actual reduction to practice of the claimed invention at a date prior to the earliest priority filing date of U.S. Publication No. 2002/0037846A1 (i.e., December 24, 1999). As required by 37 CFR § 1.131, the Declaration affirms that the acts relied upon to establish actual reduction to practice were carried out in the United States. Therefore, Applicants submit that U.S. Publication No. 2002/0037846A1 is not an effective reference against the present claims.

Objection to the Specification and Rejection of Claims 1, 3-10, 12-15, 20-30 and 38-41 Under 35 U.S.C. § 112, First Paragraph:

The Examiner has objected to the specification and rejected Claims 1, 3-10, 12-15, 20-30 and 38-41 under 35 U.S.C. § 112, first paragraph, on the basis of enablement. Specifically, the Examiner contends that the specification, while enabling for a method to inhibit airway hyperresponsiveness (AHR) in a mammal by administering to the mammal CGRP, the specification does not enable: administering *any* agent, such as a fragment of CGRP, or a homologue or analog of CGRP, or other product of drug design that binds to and activates a CGRP receptor; the use of the above-mentioned agents with CGRP RAMP; or any of these methods in conjunction with the various dependent embodiments of the claims. The Examiner asserts that the specification does not teach how to make and use the variants of CGRP. For example, the Examiner contends that a fragment of CGRP could be as little as a single amino acid. The Examiner also asserts that there is no guidance as to which amino acids of CGRP could be modified and retain CGRP activity. The Examiner further contends that in the absence of a structure of a CGRP homologue or analog, one can not make the agents. The Examiner acknowledges that at the time of the invention, the art had taught how to make some homologues and analogs of CGRP with biological activity, but the Examiner asserts that none of these biological activities is related to the claimed method.

Applicants traverse the rejection of Claims 1, 3-10, 12-15, 20-30 and 38-41 under 35 U.S.C. § 112, first paragraph. Initially, in order to expedite prosecution, Applicants have amended Claim 1 to recite the simplified list of CGRP agents of: CGRP, a fragment of CGRP having biological activity, and a homologue of CGRP having biological activity. The Examiner contends that even though, at the time of the invention, the art had taught how to make some homologues and analogs of CGRP with biological activity, none of these biological activities is related to the claimed method. In response, Applicants of course acknowledge that none of these references describe the use of CGRP to inhibit allergen-induced AHR, as this is the present invention. However, Applicants assert that it is incorrect to conclude that none of the biological activities described for CGRP homologues in the art is related to the present invention. A demonstration that a fragment or homologue of CGRP has similar biological activity to the native peptide, regardless of the standard used to compare the two moieties, is sufficient to provide at least a reasonable expectation that biologically active fragment or homologue will operate in a similar manner to the native peptide in other applications, such as the method of the present invention. When others have measured various

biochemical or physiological parameters of CGRP homologues, the comparison is made with respect to a biological activity of a native CGRP in which those investigators have an interest. CGRP binds to and activates a CGRP receptor, which results in a biological effect. If a homologue has a similar effect, one can assume that the homologue also bound to the receptor and activated that receptor in a similar manner and therefore, regardless of what tissue or what specific type of endpoint is measured, the measurement should be indicative of shared structural characteristics between the homologue and the native peptide that correlate with the function of the peptides. Given that the inventors have demonstrated that CGRP can inhibit allergen-induced AHR, it is submitted that any CGRP fragment, homologue, or even analog with substantially similar CGRP biological activity should be deemed suitable for use in the present invention.

Furthermore, the present specification teaches the following information regarding the structure and function of CGRP and fragments and homologues thereof, as known in the art at the time of the invention:

(1) Prior to the present invention, the α and β-forms of CGRP have been isolated and fully characterized by amino acid sequencing and fast atom bombardment-mass spectrometry (FABMS) (Wimalawansa, S. J., Morris, H. R., Etienne, A., Blench, I., Panico, M., and Maclntyre, I. Isolation, purification and characterization of b-hCGRP from human spinal cord, *Biochem. Biophys. Res. Commun.*, 167, 993 (1990); Steenberg, et al. FEBS Letts. 183:403 (1985)). **(see page 26, line 28 to page 27, line 5)**

(2) The nucleic acid and amino acid sequence of human CGRP are described in U.S. Patent Nos. 4,736,023 and 4,549,986, respectively, as well as methods of synthetically producing human CGRP. **(see page 27, lines 5-8)**

(3) The nucleic acid and amino acid sequences for CGRP from a variety of mammalian species can be found in public databases (see, for example, Entrez Accession Nos: AAA00500 (human CGRP from U.S. Patent No. 4,549,986); XP006209, TCHU and NP001732 (human CGRP α); XP006016, P10092, A25864 (human CGRP β); AAK16431 (mouse CGRP α); AAK06841 (mouse CGRP β); A44173 (rat CGRP β); CAB97487 (dog CGRP); and P31888 (sheep CGRP)). **(see page 27, lines 9-14)**

(4) Homology of CGRP among species is very high and the working examples of the specification show that the mouse and human CGRP peptides can be used interchangeably (i.e., the Examples show the administration of human CGRP in mice). **(see page 27, lines 17-19 and Example 3)**

(5) Amino acid positions 8-37 of CGRP has an antagonist function. **(see page 27, lines 24-26 and Example 3)**

(6) Various agonist homologues of CGRP are known in the art and are described, for example, in U.S. Patent No. 4,697,002 to Kempe (substitutions at position 36); U.S. Patent No. 4,687,839 to Kempe (D-amino acid substitutions at minimally positions 36 and 37); and U.S. Patent No. 4,530,838 to Evans et al. (substitutions at position 35). Methods for determining the biological activity of CGRP are described in these patents and can be used to evaluate other CGRP homologues. **(see page 34, line 27 to page 35, line 5)**

Both U.S. Patent No. 4,697,002 and U.S. Patent No. 4,687,839 (both referenced in the present specification as filed and in item (6) above) teach how to make several homologues of CGRP by substitutions of amino acids at the C-terminus that are taught to provide CGRP analogs having "biological activity of the same type as known CGRP" (e.g., see col. 2, lines 14-17 of the '839 patent and col. 2, lines 6-9 of the '002 patent). Similarly, U.S. Patent No. 4,530,838 (also referenced in the present specification and in item (6) above) teaches other homologues of CGRP having substitutions and derivatizations at the C-terminus that have "substantially the same biological activity" as native CGRP (see col. 2, lines 39-48). In the response filed January 29, 2003, Applicants provided two additional references. U.S. Patent No. 4,720,483 describes a variety of functional derivatized CGRP homologues. PCT Publication No. WO 89/03686 also teaches CGRP homologues (see alignment for comparison of structures on page 15, Table 2) with biological activity similar to that of the native protein. In addition, WO 89/03686 provides direction as to where in the protein substitutions have different effects on the biological activity of the protein. U.S. Patent No. 5,122,376 (see Applicants' first 1449) also describes homologues of CGRP comprising substitutions and derivatizations

With regard to the Examiner's argument that a fragment of CGRP could be a small as one amino acid, Applicants submit that this is not possible, given that the fragment was also previously required to bind to and activate a CGRP receptor and is currently required to have CGRP biological

activity. A single amino acid is clearly not a sufficient structure to bind to and activate a CGRP receptor or to have the biological activity of native CGRP. Moreover, the level of knowledge in the art regarding the structure of CGRP and what modifications may be tolerated to preserve or destroy the function of the peptide readily allows one of skill in the art to make and use biologically active fragments of CGRP with a reasonable expectation of success and without undue experimentation.

It is further noted that the specification teaches that CGRP activity can be detected by measurement of the binding of CGRP to a receptor and to measuring one or more activities in a cell expressing the receptor, including, but not limited to, increasing cAMP, increasing intracellular calcium mobilization and phosphorylation of the receptor. The specification also clearly teaches how to confirm the activity of CGRP or a homologue or fragment thereof with regard to the inhibition of allergen-induced AHR.

Finally, with regard to the Examiner's citation of Zhu et al., while the Examiner states that Zhu et al. "teach that calcitonin gene-related peptide (CGRP) may play different physiological and pathophysiological roles in airway regulation in different species such as horse, human,[sic] Sprague-Dawley rat, and mouse", Applicants respectfully submit that this is not an accurate representation of the teachings of Zhu et al. Zhu et al. state in the Discussion that "peptidergic nerves may play different physiological and pathophysiological roles in airway regulation in different species" (emphasis added). The study of Zhu et al. is directed primarily to the effect of *capsaicin* on sensory nerve fibers and they conclude that sensory neuropeptides such as SP or CGRP released from *capsaicin-sensitive* nerves have no *direct* effect on smooth muscle tone, although the possibility of indirect effects is not ruled out. Applicants submit that the conclusions of Zhu et al. do not merit the expansion of the findings to the conclusions proposed by the Examiner.

In summary, given the volume of information provided in the specification and known in the art at the time of the invention about the structure of CGRP and of various substitutions and derivatizations that can be made to the protein and preserve the function of the native protein, Applicants fail to understand how the Examiner views the provision of biologically active CGRP fragments and homologues as unpredictable.

In view of the foregoing discussion, Applicants respectfully request that the Examiner withdraw the rejection of Claims 1, 3-10, 12-15, 20-30 and 38-41 under 35 U.S.C. § 112, first paragraph.

Objection to the Specification and Rejection of Claims 1, 3-10, 12-15, 20-30 and 38-41 Under 35 U.S.C. § 112, First Paragraph:

The Examiner has objected to the specification and rejected Claims 1, 3-10, 12-15, 20-30 and 38-41 under 35 U.S.C. § 112, first paragraph, on the basis of written description. Specifically, the Examiner contends that the specification does not provide an adequate written description of the method to inhibit AHR comprising administering *any* agent that binds to and activates a CGRP receptor as claimed. The Examiner asserts that there is no description of the structure of a fragment of CGRP or a homologue or analog of CGRP or a product of drug design that demonstrates that Applicants were in possession of a representative number of species to describe the genus. The rejection essentially reiterates much of the argument from the enablement rejection above.

Applicants traverse the rejection of Claims 1, 3-10, 12-15, 20-30 and 38-41 under 35 U.S.C. § 112, first paragraph. Initially, Applicants refer to the discussion under the enablement rejection above and particularly, the provision in the specification and in the art at the time of the invention a substantial body of information regarding the structure of CGRP and what amino acids may be substituted and/or derivatized, while retaining or destroying the biological activity of the peptide. The issue at hand is whether one of skill in the art would reasonably conclude from the specification that the disclosure provides a representative number of species within the claimed genus to demonstrate possession of the invention. Applicants strongly traverse the Examiner's contention that there is no description of the structure of a fragment of CGRP or a homologue or analog of CGRP. To the contrary, Applicants submit that clearly, referring again to the discussion above, the specification and the art have provided teachings of the structure of native CGRP from a variety of animal species (e.g., human CGRP-α and CGRP-β), as well as numerous examples of the structure of homologues and analogs of CGRP, including specific amino acid residues that can be substituted or derivatized while maintaining the similar biological activity of the native CGRP. In addition, the specification provides a teaching of a homologue that does not have CGRP biological activity,

providing additional information about where amino acid modifications can not be readily tolerated. Applicants submit that the evidence of record shows that there was significant knowledge in the art at the time of the invention regarding the structure correlated with the function of CGRP.

Moreover, the specification does not merely allude to the structure of CGRP and fragments and homologues thereof, as the Examiner asserts, but provides specific definitions of such agents and several references to the known structure of the native CGRP peptides as well as to various homologues thereof. Applicants do not believe that the written description requirement obligates Applicants to test each and every homologue of CGRP in the claimed method. Instead, given that the art already provides multiple biologically active CGRP homologues and guidance to produce the same, it is believed that this is sufficient to demonstrate possession of the invention as claimed.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of Claims 1, 3-10, 12-15, 20-30 and 38-41 under 35 U.S.C. § 112, first paragraph.

Rejection of Claims 1, 3-5, 8-9, 20, 23, 26, 29 and 38 Under 35 U.S.C. § 103:

The Examiner has rejected Claims 1, 3-5, 8-9, 20, 23, 26, 29 and 38 under 35 U.S.C. § 103, contending that these claims are not patentable over Nagase et al. in view of Hoshino et al. or Elwood et al. The Examiner contends that Hoshino et al. teach that allergen-induced bronchoconstriction in the airway in guinea pigs sensitized to an allergen is accompanied by AHR and infiltration into the airway lumen of inflammatory cells. The Examiner further contends that Elwood et al. teach that bronchial hyperresponsiveness and increase in eosinophils in BALF of rats sensitized to allergen such as ovalbumin has many characteristics of human allergen-induced bronchial hyperresponsiveness. The Examiner contends that Nagase et al. teach a method of inhibiting hyperpnea (dry air) induced AHR in a mammal such as a guinea pig by administering to the guinea pig CGRP. The Examiner states that the physiology of airway constriction and AHR in hyperpnea induced AHR is equivalent to exercise-induced asthma in humans or allergen induced airway constriction as taught by Hoshino et al. Therefore, the Examiner concludes that the combined teachings of Nagase et al., Hoshino et al., and Elwood et al. provide clear direction, motivation and expectation of success at treating AHR using CGRP.

Applicants traverse the rejection of Claims 1, 3-5, 8-9, 20, 23, 26, 29 and 38 under 35 U.S.C. § 103. Applicants initially note that the Examiner has stated that the claimed invention differs from the teachings of Nagase et al. in that the method inhibits allergen-induced AHR in a mammal comprising[sic] administering to a mammal an agent such as CGRP that binds to and activates a CGRP receptor. Applicants submit that the Examiner seems to have listed nearly all of the elements of the claimed invention (i.e., inhibition of allergen-induced AHR, administration to a mammal of an agent that binds to and activates a CGRP receptor). Therefore, it appears that the Examiner acknowledges critical deficiencies in the teachings of Nagase et al.

In any event, Applicants maintain the following prior argument against the Examiner's reasons for including Nagase et al. in the combination of references. The model studied by Nagase et al. mimics a type of airway hyperresponsiveness that results from deep and rapid breathing, particularly of cold, dry air such as occurs in exercise-induced asthma (Nagase et al., page 1551, first paragraph). Applicants submit that this *hyperpnea-induced* AHR has a <u>different cause</u> and <u>different general pathophysiology</u> than allergen-induced AHR, and these differences are supported by Nagase et al. and the present specification. <u>As discussed in Nagase et al.</u>, hyperpnea challenge causes constriction a few moments after challenge, resolves spontaneously, is reproducible upon identical challenge, and is believed to be associated with tachykinins and sensory nerves (e.g., see Nagase et al., paragraph spanning page 1554-1555). In contrast, <u>as discussed in the present specification</u>, allergen-induced AHR is induced by an initial sensitization to, followed by subsequent challenge with, an allergen, and is associated with an immune (IgE) response, a dependence on a Th2-type response and an eosinophil response, and is both a marked and evolving hyperresponsiveness of the airways (see page 12, lines 1-8 of the specification).

One of skill in the art will understand, and indeed it is clear from the discussion of the respective airway conditions in Nagase et al. and the present specification, that hyperpnea-induced AHR and allergen induced AHR are <u>not</u> equivalent conditions, and therefore that a method to treat hyperpnea-induced AHR <u>is not equivalent</u> to a method to treat allergen-induced AHR, nor can the usefulness of a compound in hyperpnea-induced AHR be simply extrapolated be useful in allergen-induced AHR.

In further support of this point, Applicants enclose herewith two documents. The first reference (see Solway J, in Asthma, Barnes P.J., Grunstein MM, Less AR, and Wodcock AJ, Lippincott-Raven Publishers, Philadelphia, 1997, pp. 1128-1129) is an excerpt from a chapter entitled "Hyperpnea-Induced Bronchoconstriction" from a reference for asthma, which teaches that hyperpnea-induced bronchoconstriction (i.e., hyperpnea-induced AHR) is secondary to (a) hyperemia edema, or (b) hyperosmolarity due to water loss (see page 1128, column 2 to page 1129, middle of column 2). In contrast, as discussed in the specification, allergen-induced AHR is secondary to inflammation. The second reference (see Ingenito et al., *Am. Rev. Respir. Dis.* 146:1315-1319, 1992), *directly* compares antigen(allergen)-induced AHR to hyperpnea-induced AHR and teaches that there are significant differences in constriction reversal, BAL total proteins, inflammation, and volume history responses between the two types of AHR, showing that the conditions are different. Referring to the paragraph bridging page 1317 to page 1318 of Ingenito et al., the reference teaches that:

> the "potential importance of differentiating bronchoconstriction associated with, or arising from, inflammation and that related to transient airway smooth-muscle constriction is well recognized inasmuch as both therapy and prognosis differ. Indirect evidence from both human and animal studies supports the assertion that these represent different pathophysiological states."

The paragraph goes on to provide some detailed discussion of specific differences between the types of bronchoconstriction and concludes: "[t]hus the two stimuli appear to produce obstructive physiology by different mechanisms, one involving inflammation." These references demonstrate that, at the time of the invention, hyperpnea-induced AHR and allergen-induced AHR were known to be pathophysiologically different conditions.

With regard to the combination of references cited by the Examiner, in response to Applicants' argument above, the Examiner contends that hyperpnea-induced AHR is equivalent to exercise-induced asthma in humans and to allergen-induced airway hyperresponsiveness. Applicants note that the Examiner does not specifically address or attempt to rebut the clear distinctions between hyperpnea-induced AHR as discussed by Nagase et al. and allergen-induced AHR as discussed in the present specification, all of which were clearly presented in the last response. Instead, the

Examiner's response points to Hoshino et al. as providing a teaching that allergen-induced AHR is equivalent to hyperpnea-induced AHR (see Office Action, page 14, second full paragraph). However, Applicants are unable to find in Hoshino et al. any teaching that allergen-induced AHR is equivalent to hyperpnea-induced AHR or to exercise-induced AHR. Indeed, in the Office Action, the Examiner has only characterized Hoshino et al. as teaching that allergen-induced bronchoconstriction in guinea pigs sensitized to allergen is accompanied by AHR and infiltration into the airway lumen of inflammatory cells such as eosinophils and neutrophils. As discussed above, these are characteristics of allergen-induced AHR as discussed in the specification, but do not relate to hyperpnea-induced AHR, nor is there any teaching in any of the cited references, including Hoshino et al., of such an equivalence as proposed by the Examiner. Hoshino et al. is directed to the study of the anti-allergy drug, ketotifen on atopic asthma (i.e., all of the patients in these studies were atopic, meaning that they suffered from allergen-induced asthma). Hoshino et al. discuss only atopic asthma; Applicants find no discussion of exercise-induced AHR in Hoshino et al.

Similarly, Applicants find no discussion of any alleged correlation between exercise-induced AHR and allergen-induced AHR in Elwood et al. Elwood et al., as the Examiner contends, is directed to the study of allergen-induced bronchial hyperresponsiveness. Elwood et al. study the effects of allergen challenge on bronchial responsiveness and the influx of inflammatory cells into the lungs and airways. Elwood et al. find correlations between the increases in inflammatory cells in the lungs and AHR. However, Elwood et al. do not provide any teachings regarding exercise-induced asthma or exercise-induced AHR. Indeed, referring to the paragraph spanning the columns on page 959, one can clearly see that the only comparison made between rats and humans is with regard to allergic airway inflammatory responses.

In order to establish a case of obviousness, a combination of references must: (1) teach each and every element of the claimed invention; (2) provide a motivation to make the combination and arrive at the claimed invention; and (3) provide a reasonable expectation of success at being able to practice the claimed invention.

Applicants submit that not only has the Examiner failed to provide a combination of references that teach the administration of CGRP, fragment or homologue thereof to inhibit allergen-

induced AHR, the Examiner has also failed to provide any motivation in the references or in the art to combine the references and has failed to provide any reasonable expectation of success at making the combination. The Examiner has not pointed to any teaching in any of the references that establishes an equivalency of hyperpnea-induced AHR and allergen-induced AHR and has not pointed to any teaching that establishes that administration of CGRP inhibits allergen-induced AHR. Applicants have now provided additional evidence that hyperpnea-induced AHR and allergen-induced AHR are <u>not</u> equivalent conditions, which directly rebuts the Examiner's position. Indeed, it would be impossible to predict from the teachings of Nagase et al. any effect of CGRP on allergen-induced AHR and the references of Elwood et al. and Hoshino et al. do not discuss CGRP and therefore can provide no expectation of success at using this agent to treat allergen-induced AHR. If the Examiner intends to maintain the position that hyperpnea-induced AHR is equivalent to allergen-induced AHR, then Applicants respectfully request that the Examiner either point to specific support in the art for this contention or provide a statement in the form of a Declaration under the Examiner's signature.

Finally, the Examiner characterizes Applicants' prior response as concluding that Nagase et al. saw no effect on airway constriction from the administration of CGRP. Wishing to clarify the record, what Applicants stated is that <u>in the methacholine and endothelin-1 experiments</u>, which the Examiner referenced in part to form the rejection, Nagase et al. saw no effect of the administration of CGRP on airway constriction. This argument is a direct rebuttal, supported by Fig. 5 of Nagase et al., of the Examiner's contention that Nagase et al. show an effect of CGRP on methacholine and endothelin-1 induced constriction, a contention that is repeated in the current Office Action. It is again respectfully noted that Figs. 2 and 3 of Nagase et al. do not relate to methacholine or endothelin-1 challenge and that Fig. 5 shows that these compounds had no effect on airway constriction in the animals.

In view of the foregoing discussion, Applicants respectfully request that the Examiner withdraw the rejection of Claims 1, 3-5, 8-9, 20, 23, 26, 29 and 38 under 35 U.S.C. § 103.

<u>Rejection of Claims 1, 3-10, 21-24, 27, 29-30 and 40 Under 35 U.S.C. § 103</u>:

The Examiner has rejected Claims 1, 3-10, 21-24, 27, 29-30 and 40 under 35 U.S.C. § 103, contending that these claims are unpatentable over Nagase et al. in view of Hoshino et al., Elwood et al. and U.S. Patent No. 5,858,978 or U.S. Patent No. 5,635,478. Specifically, the Examiner reiterates the arguments in view of Nagase et al., Hoshino et al. and Elwood et al. as discussed above, and further contends that the '978 patent and the '478 patent teach a method of using CGRP and homologues thereof in a method of inhibiting acute or chronic inflammatory conditions such as asthma, which is associated with AHR. Therefore, the Examiner asserts that it would be obvious to substitute the CGRP taught by Nagase et al. for the CGRP homologue in combination with other agents as taught by the '978 or '478 patents to inhibit allergen-induced AHR. Applicants prior arguments are addressed by the Examiner as in the rejection under § 103 above.

Applicants traverse the rejection of Claims 1, 3-10, 21-24, 27, 29-30 and 40 under 35 U.S.C. § 103. Applicants refer to the discussion regarding the combination of Nagase et al. with Hoshino et al. and Elwood et al. above, and further contend that neither of the '978 patent or the '478 patent make up for the deficiencies of the first combination. As previously discussed, The '978 patent and the '478 patent are directed to the use of CGRP to ameliorate inflammatory conditions by inhibiting the release of the proinflammatory cytokines, IL-1, or IL-1 and IL-2, from immune cells such as macrophages and lymphocytes. The use of CGRP is disclosed by these patents as being useful for the treatment of a wide variety of diseases, of which asthma is only one. Moreover, for the reasons of record, Applicants submit that asthma and AHR are not one in the same condition, but rather conditions that can be associated with one another, and neither of the '978 patent or the '478 patent teach or suggest treating AHR. Finally, Applicants again submit that not only do the cited patents fail to *specifically* teach the treatment of allergen-induced AHR, the patents teach the use of CGRP to treat inflammation in such an incredibly broad variety of diseases and conditions that the reference as a whole *fails* to teach or suggest the claimed method for treatment of allergen-induced AHR with *sufficient specificity* to teach or suggest the claimed invention. As previously discussed, treatment of inflammation does not lead one of skill in the art to contemplate the treatment of AHR, because treatment of inflammation can occur in the absence of an effect on AHR, as these are separate conditions. Second, even with an association between inflammation and allergen-induced AHR, Applicants submit that the suggestion to inhibit the release of IL-1 or IL-1 and IL-2 in a patient with

allergen-induced AHR or indeed, any allergic inflammation, including allergic asthma, is not consistent with, and in fact is *contrary to*, what is known about allergic inflammation by those of skill in the art, also previously discussed. This would represent a *teaching away* from the present invention.

The Examiner seems to continue to cite these patents as providing a teaching of administration of CGRP with other agents, such as cortisone or phosphodiesterase inhibitor. The Examiner again equates hyperpnea-induced AHR with allergen-induced AHR, but as discussed above, Applicants assert that the Examiner has not provided any teaching in any of the references, alone or in combination, that these conditions are equivalent. Furthermore, Applicants submit that there is no motivation to combine the '978 and '478 patents with Nagase et al., since the patents are directed to the inhibition of IL-1 or IL-1 and IL-2, whereas Nagase et al. is directed to hyperpnea-induced AHR, which is not related to these inflammatory cytokines. Therefore, Applicants submit that this combination of five references fails to teach the claimed invention, fails to provide motivation to arrive at the claimed invention, and fails to provide any expectation of success at making or using the claimed invention.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of Claims 1, 3-10, 21-24, 27, 29-30 and 40 under 35 U.S.C. § 103.

Rejection of Claims 25 and 27 Under 35 U.S.C. § 103:

The Examiner has rejected Claims 25 and 27 under 35 U.S.C. § 103, contending that these claims are unpatentable over Nagase et al. in view of Hoshino et al. and Elwood et al., as applied in the rejections above, and in further view of Suissa et al. Specifically, the Examiner refers to the rejections above, and adds that Suissa et al. teach leukotriene antagonists for the treatment of asthma, and that it would therefore be obvious to combine Suissa et al. with the remaining references to arrive at Claims 25 and 27. Applicants prior arguments are addressed by the Examiner as in the rejection under § 103 above.

Applicants traverse the rejection of Claims 25 and 27 under 35 U.S.C. § 103. Applicants refer to and reiterate the arguments above with regard to the combination of Nagase et al., Hoshino et al. and Elwood et al. and further submit that the combination of any or all of these references with

Suissa et al. does not remedy the deficiencies of the primary references. Suissa et al. teach the use of leukotriene receptor antagonists and beta agonists to treat asthma, and do not teach or suggest using CGRP to treat allergen-induced airway hyperresponsiveness. Suissa et al. do not teach or suggest that exercise-induced AHR and allergen-induced AHR are equivalent. Therefore, there is absolutely no motivation in Suissa et al., or in the remaining references in the combination as discussed above, to form this combination as the Examiner has done.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of Claims 25 and 27 under 35 U.S.C. § 103.

Rejection of Claim 27 Under 35 U.S.C. § 103:

The Examiner has rejected Claim 27 under 35 U.S.C. § 103, contending that these claims are unpatentable over Nagase et al., in view of Hoshino et al. and Elwood et al. as previously discussed in the rejections above, and in further view of Drazen et al. or Abraham et al., or Abdelaziz et al., or Barnes et al., or Hoshino et al. Specifically, the Examiner refers to the prior rejection in view of the Nagase et al., Hoshino et al. and Elwood et al. and adds that (1) Drazen et al. teach that leukotriene receptor antagonists are safe and effective asthma treatment; (2) Abraham et al. teach agents such as cromolyn sodium and beta 2 mimetic reproterol hydrochloride in combination gives better protection against post-antigen-induced AHR; (3) Abdelaziz et al. teach agents such as Nedocromil can reduce AHR; (4) Barnes et al. teach agents such as theophylline for treatment of asthma; (5) Hoshino et al. teach an agent such as ketotifen is beneficial for inhibiting activated eosinophils and T cell infiltration into the airways. The Examiner asserts that it would have been obvious to combine the teachings above to arrive at a teaching of administration of CGRP with other agents. The Examiner addresses Applicants' prior arguments against this rejection in the same manner as in the first rejection above.

Applicants traverse the rejection of Claim 27 under 35 U.S.C. § 103. Applicants refer to and reiterate the arguments above with regard to the combination of Nagase et al., Hoshino et al. and Elwood et al. and further submit that the combination of any or all of these references with any of Drazen et al., Abraham et al., Abdelaziz et al., or Barnes et al., alone or in combination, does not remedy the deficiencies of the primary references. None of Drazen et al., Abraham et al., Abdelaziz

et al. or Barnes et al. teach, suggest, or provide motivation to do anything but use a completely different agent for a condition specified in that reference. Similarly, none of these references is directed to the use of CGRP for any reason and so none of these references can remedy the lack of teaching, motivation, or expectation of success in the combination of references. Furthermore, none of these additional references teach that hyperpnea-induced AHR and allergen-induced AHR are equivalent conditions.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of Claim 27 under 35 U.S.C., § 103.

<u>Rejection of Claims 12-14 Under 35 U.S.C. § 103</u>:

The Examiner has rejected Claims 12-14 under 35 U.S.C. § 103, contending that these claims are unpatentable over Nagase et al., in view of Hoshino et al. and Elwood et al. for the reasons set forth above, and in further view of Cadieux et al. Specifically, the Examiner refers to the prior rejection in view of Nagase et al., Hoshino et al. and Elwood et al. and adds that Cadieux et al. teach administering an agent such as CGRP at a given concentration. The Examiner contends it would have been obvious to combine the teachings and arrive at the invention claimed in Claims 12-14.

Applicants traverse the rejection of Claims 12-14 under 35 U.S.C. § 103. Applicants again refer to and reiterate the arguments above with regard to the combination of Nagase et al., Hoshino et al. and Elwood et al. and further submit that the combination of any or all of these references with Cadieux et al., does not remedy the deficiencies of the primary references. The Examiner seems to cite Cadieux et al. for a teaching of various dosages of CGRP that could be administered. As discussed previously, Cadieux et al. teach that CGRP inhibits bronchoconstriction that is <u>not</u> induced by allergen (similar to Nagase et al.), and further teach that CGRP was found to be *ineffective* against the constriction in inflammatory conditions. Indeed, the Examiner seems to acknowledge this latter point, which is a clear teaching away from the present invention, yet the Examiner still makes the combination following this acknowledgment. However, it appears that the combination is based primarily on the teachings of the first three references, which Applicants have discussed in detail above. Applicants submit that not only does the combination of any of the primary references with Cadieux et al. fail to teach or suggest the claimed method, the combination does not motivate one

of skill in the art to arrive at the invention and provides no expectation of success because Cadieux et al. actually *dissuade* one of skill in the art from arriving at the claimed method by concluding that the use of CGRP in inflammatory conditions is inoperable. This is effectively a *teaching away* from the claimed invention. The teaching of doses by Cadieux et al. does not remedy any deficiencies of the combination of references.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of Claims 12-14 under 35 U.S.C. § 103.

Rejection of Claims 15, 28, 39 and 41 Under 35 U.S.C. § 103:

The Examiner has rejected Claims 15, 28, 39 and 41 under 35 U.S.C. § 103, contending that these claims are unpatentable over Nagase et al., in view of Hoshino et al. and Elwood et al., and in further view of WO 98/03534. Specifically, the Examiner refers to the prior rejection in view of Nagase et al., Hoshino et al. and Elwood et al. and adds that the WO 98/03534 reference teach various CGRP agonists and antagonists such as CGRP-RCF analog and fragments thereof for treating allergies. The Examiner asserts that it would have been obvious to substitute the analog such as CGRP-RCF of WO 98/03534 in the method of Nagase et al., Hoshino et al. and Elwood et al.

Applicants traverse the Examiner's rejection of Claims 15, 28, 39 and 41 under 35 U.S.C. § 103. Applicants again refer to and reiterate the arguments above with regard to the combination of Nagase et al., Hoshino et al. and Elwood et al. and further submit that the combination of any or all of these references with WO 98/03534 does not remedy the deficiencies of the primary references. The Examiner asserts that the CGRP-RCF of WO 98/03534 is a CGRP agonist or antagonist. Applicants disagree. WO 98/03534 teach that CGRP-RCF is a *CGRP receptor component factor*, a 148 amino acid peptide which confers CGRP responsiveness to a CGRP receptor expressed by oocytes, apparently by allowing expression of the receptor. CGRP-RCF is not CGRP or an agonist or antagonist thereof. Page 16, lines 14-20 of WO 98/03534 teach that the receptor component factor refer to molecules *other than* CGRP receptor ligands or CGRP receptors. WO 98/03534 teach that CGRP-RCF might be useful to treat large number of virtually unrelated diseases. WO 98/03534 does not teach or suggest the use of CGRP or any compound to inhibit allergen-induced AHR and

can not remedy the deficiencies of the combination of Nagase et al., Hoshino et al., and Elwood et al. as discussed above.

In view of the foregoing discussion, Applicants respectfully request that the Examiner withdraw the rejection of Claims 15, 28, 39 and 41 under 35 U.S.C. § 103.

Applicants have attempted to address all of the concerns as set forth in the April 22 Office Action. In the event that the Examiner has any additional concerns regarding Applicants' position, the Examiner is invited to contact the undersigned agent at (303) 863-9700.

Respectfully submitted,

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■ 78 ■

Hyperpnea-Induced Bronchoconstriction

Julian Solway

As discussed in Chapter 77, exercise is a common if not universal precipitant of bronchoconstriction in patients with asthma. A great breakthrough in understanding the pathophysiology of exercise-induced bronchoconstriction came when a series of studies established that hyperventilation, induced by exercise, constitutes the key feature of exercise that generates its bronchoprovocative stimulus (1–8). This realization led to the development of isocapnic voluntary hyperventilation as a laboratory near-equivalent of exercise as a bronchoprovocative stimulus. Isocapnic voluntary hyperventilation in the laboratory setting facilitates experimental control of a wide range of potential variables (such as inspired air conditions, intensity of hyperpnea, breathing pattern, etc.) that are less easily controlled during exercise. Studies incorporating isocapnic voluntary hyperventilation have, therefore, shed light on the factors that determine the severity of exercise-induced bronchoconstriction, and have provided some insight into the pathophysiological mechanisms responsible for this phenomenon. It is true that exercise induces hormonal responses that do not occur during isocapnic voluntary hyperventilation, such as adrenal catecholamine release, and generation of prostanoid mediators that appear responsible for the refractory period after exercise, as discussed in Chapter 77 (9–11). Yet, the weight of evidence strongly supports a view that these specifically exercise-related phenomena simply modulate the same fundamental provocation and airway response induced by the purer challenge of isocapnic voluntary hyperventilation without exercise (12,13).

This chapter will examine the airway response to isocapnic voluntary hyperventilation. Consideration will be given to how the physical consequences of hyperventilation promote and simultaneously inhibit bronchoconstriction, examine the airway wall components that affect airway narrowing, and evaluate the biochemical pathways involved in this response. Many of these important issues remain incompletely resolved. Yet, there are now sufficient data from studies in humans and in relevant animal models to allow the formulation of a theory of the pathophysiology of this common phenomenon in asthma.

WHAT IS ISOCAPNIC VOLUNTARY HYPERVENTILATION?

To perform isocapnic voluntary hyperventilation, an individual breathes conditioned air through a mouthpiece attached to a nonrebreathing valve. Inspired air is directed through a refrigerated or warmed heat exchanger that adjusts its temperature, and inspired air humidity (during warm air breathing) is controlled by passing dry gas through a bubble humidifier at a known bath temper-

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tain. It has been suggested that these agents inhibit mast cell degranulation; yet, potent antihistamines offer only modest protection against exercise-induced bronchoconstriction (49–53). Alternatively, cromolyn and nedocromil may exert their effects by suppressing the activation of sensory C-fibers that innervate the airways (54,55). These sensory nerves can release tachykinins that constrict airway smooth muscle. A recent report suggests that a novel antagonist (FK888) of NK-1 tachykinin receptors can blunt the constrictor response to dry gas hyperpnea partially (56–60). However, the role of sensory C-fibers in HIB has not been assessed completely, since there are no reports of the effects of NK-2 tachykinin receptor antagonists on HIB in asthma. NK-2 receptors are more likely the important species if sensory nerve tachykinins mediate airway smooth muscle contraction in HIB (56). Interestingly, the influence of cromolyn and nedocromil might be partially attributable to inhibition of tachykinin effects, possibly through receptor blockade (61–63). Atropine has only minor influence on HIB in asthma, indicating that this response is not primarily mediated through cholinergic neurotransmission (64,65).

Inhibitors of leukotriene synthesis (including 5-lipoxygenase inhibitors and antagonists of 5-lipoxygenase activating protein) and leukotriene D4 (LTD4) receptor antagonists both blunt HIB in many, though not all, asthmatic subjects (14,66–68) (Fig. 8). There is reason to suspect that the airways of asthmatic individuals contain elevated levels of LTD4. Activated human eosinophils, which infiltrate asthmatic airways, secrete LTC4, which is converted readily to LTD4 through the action of γ-glutamyl transpeptidase (69,70). However, direct evidence that peptidoleukotrienes are released by airway cells acutely during or after dry gas hyperpnea is lacking. Indeed, leukotriene E4 (the main metabolite of LTD4) is recovered in increased quantity in urine of acutely ill asthmatic subjects presenting for emergency treatment or acutely after allergen challenge (71,72). Yet, most studies report no acute increase in urinary LTE4 excretion following exercise-induced bronchoconstriction (72–74). Thus, as to be discussed, it may be that the importance of LTD4 in HIB stems from some other effect than directly causing acute airway smooth muscle contraction.

Four other agents blunt HIB through unknown mechanisms. Furosemide, when given as aerosol pretreatment, can inhibit HIB in asthmatic subjects (75,76). Heparin sodium aerosol also inhibits exercise-induced bronchoconstriction, possibly by inhibiting mediator release from mast cells (77–79). Cyclooxygenase inhibitors have also exhibited minor inhibitory effects on exercise-induced bronchoconstriction (52). Finally, α-adrenergic agonists have been shown to blunt HIB, an effect that has implicated a role of the airway vasculature in generating airflow obstruction after dry gas hyperpnea (80,81).

PATHOPHYSIOLOGY OF HYPERPNEA-INDUCED BRONCHOCONSTRICTION

Nature of the Physical Stimulus that Provokes HIB

The characteristic features of HIB in human asthmatic subjects suggest pathophysiologic mechanisms that underlie this response. The striking dependence of HIB on inspired gas temperature and humidity clearly indicates that some physical consequence of dry gas hyperpnea—airway cooling and/or airway drying—represents a key facet of the hyperventilation-induced stimulus to HIB. Theoretical and experimental studies conclusively demonstrate that heat and water are transferred from the airway walls to cool, dry inspired gas, as the inspirate flows over the bronchial surface along its course from airway opening to alveoli (82–84). Most airstream warming and humidification occur in the central airways, but the deeper airways also participate when $\dot{V}_E$ is increased. Transfer of heat from the airways to inspirate occurs through convection and evaporation, and tends to cool the airways. In turn, heat can be replenished to the airways from circulatory heat sources (including the pulmonary and bronchial circulations), so the net change in airway wall temperature at any depth into the respiratory path reflects a local balance of heat loss and heat replenishment (85). Some heat is also recovered by the bronchi during exhalation, as warm, humid alveolar gas loses some of its energy and water content to the relatively cool bronchial walls. Similar principles govern the balance of water transfers between airway walls and inspired gas during hyperpnea. The relatively dry inspirate acquires water through evaporation of bronchial surface fluid, such that the airstream contains 44 mg/L (100% water vapor saturation at 37°C) by the time it reaches the alveoli. This water loss tends to dry the airways, an effect balanced partially by water replenishment from the bronchial circulation and by minor recovery of water from exhaled alveolar gas through condensation. Of course, when hyperpnea is discontinued and quiet breathing restored, both hyperpnea-induced cooling and drying can be expected to resolve over a time course determined by the rewarming and rehydrating mechanisms noted earlier.

The dual physical consequences of dry gas hyperpnea—airway cooling and airway drying—have prompted competing hypotheses about the physical stimulus that provokes HIB. Some investigators focused on the airway cooling effects of warming and humidifying cool, dry inspired air, while others stress the importance of water loss-induced osmolarity changes of the bronchial lining fluid. Proponents of the importance of airway heat loss and posthyperpnea rewarming have focused on the possible vascular effects of such temperature changes, noting the prominent vasodilation that occurs in some systemic vessels following rewarming after cold exposure (86–92). They argue that hyperemia and/or edema swell the airway

walls as they rewarm following the cessation of hyperpnea-induced airway cooling. In support of this pathogenetic view are the findings that pretreatment with vasoconstrictor α-adrenergic agonists blunt HIB, and that maneuvers which may diminish airway-wall cooling (... as administration of cromolyn sodium, furosemide inhalation, or vascular volume redistribution by inflation of antishock trousers) also blunt HIB (80,81,88,92–94). Note also that the characteristic posthyperpnea time course of airflow obstruction is easily explained if posthyperpnea rewarming and vascular responses to rewarming cause airflow obstruction. However, a number of findings from studies in animals and humans cast doubt that rewarming-induced bronchovascular responses account entirely for HIB. First, when measured in animals, bronchial blood flow increases *during* cold gas hyperventilation and falls during the posthyperpnea period; direct evidence for rewarming-induced hyperemia within the bronchial circulation is lacking (95–97). Also, interruption of the bronchial circulation has no effect on the airflow obstruction induced by insufflation of dry gas into the canine lung periphery (98). Dry gas hyperpnea does induce bronchovascular hyperpermeability that could lead to airway edema in guinea pigs, though there is little correlation between the magnitudes and axial distributions of airway narrowing and bronchovascular hyperpermeability in this model (30). Second, intra-airway airstream temperature reaches a steady axial profile within about two minutes following dry-gas hyperpnea, suggesting that airway-wall temperatures also cool to their lowest values quickly (84). It is, then, difficult to reconcile how extending the duration of dry-gas hyperpnea worsens HIB if airway-wall temperatures change no ... during extended hyperpnea. (Rewarming in the posthyperpnea period should start from the same temperature, for any hyperpnea duration greater than two minutes.) Finally, cooling of the canine peripheral airways by perfusing the pulmonary artery with cooled blood reduces their postinsufflation constrictor response to dry-gas insufflation (37). This finding may have relevance for human asthma, since a recent report illustrates other similarities between effects of dry-gas insufflation in the peripheral airways of lungs from normal dogs or asthmatic humans (99).

The notion that hyperpnea-induced airway drying constitutes the prime physical stimulus for HIB is the principal competing view (100–103). Ample evidence from intra-airway measurements of airstream temperature and water vapor indicates that hyperpnea-induced bronchial water losses can be substantial (83,95). Furthermore, the water replenishment mechanisms that oppose airway drying seem incapable of maintaining full airway-wall hydration, since breathing dry gas at even low $\dot{V}_E$ can increase airway lining fluid osmolarity and can lead to overt airway dessication and inflammation when dry-gas exposure is sufficiently severe and prolonged (104–107). Inhalation of hyperosmolar aerosols can routinely provoke bronchoconstriction in asthmatic subjects, presumably through a mechanism that depends on altered airway surface osmolarity (103). Also, there are hyperosmolarity-responsive cells within the airways that represent potential sensors of hyperpnea-induced osmolarity changes, as discussed further on. Finally, in contrast to its probable lack of influence on airway-wall temperature, extending the duration of dry-gas hyperpnea should increase airway mucosal drying with time. If increased airway mucosal osmolarity constitutes the physical stimulus that provokes HIB, then this effect could explain how extending the duration of dry-gas hyperpnea worsens the severity of HIB. On the other hand, the "water loss" hypothesis does not itself explain why the major portion of airflow obstruction follows cessation of hyperpnea (though it could account for the minor obstruction sometimes seen during hyperpnea), and it does not explain the influence of vasoconstrictors on HIB. On balance, though, it seems most likely that hyperpnea-induced osmolarity changes do represent the key physical stimulus generated by isocapnic hyperventilation, while airway cooling induced by dry-gas hyperpnea may play a more modulatory role.

Transduction of the Physical Stimulus into Airflow Obstruction

If hyperosmolarity of the airway lining fluid, and perhaps the airway wall itself, constitute the most important physical stimulus to bronchoconstriction during HIB, what is the cell species that transduces this physical stimulus into a biologic response? Two cells resident within the airway wall have emerged as the most likely candidates as sensors of hyperpnea-induced mucosal hyperosmolarity: mast cells and sensory C-fiber neurons. Mast cells exposed to hyperosmolar environment can release a variety of preformed and newly synthesized mediators *in vitro*, though this effect is less certain *in vivo* (108–110). Some of these mast cell products are known bronchoconstrictors, including histamine and LTD4, both of which have been implicated in HIB by the effects of pharmacologic inhibitors of their synthesis or action. On the other hand, studies intended to determine whether dry-gas hyperpnea causes mast cell mediator release have yielded inconsistent and contradictory results (111–115). Furthermore, even potent antihistamines have only a minor inhibitory effect on HIB, and though LTD4 antagonists or synthesis inhibitors blunt HIB in some subjects, their effects are nonuniform among asthmatic subjects, and there is evidence against the acute release of peptidoleukotrienes in the perihyperpnea period (51–53). Together, these data suggest that mast cells are not the principal species that transduces hyperpnea-induced hyperosmolarity into HIB.

Sensory C-fiber neurons innervate the airways of humans and animals, and sense a wide range of physical and

R lationship among Mediators, Inflammation, and Volume History with Antigen versus Hyp rpnea Challenge in Guinea Pigs[1–3]

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SUMMARY Paralyzed mechanically ventilated guinea pigs constricted to a similar degree by either
isocapnic hyperpnea or antigen challenge display significantly different lung resistance (RL) vol-
ume history responses to a deep breath. We compared bronchoalveolar lavage (BAL) mediator pro-
files, BAL total protein concentrations, and tissue histopathology of antigen-constricted (AC),
hyperpnea-constricted (HC), and control guinea pigs to determine whether patterns of volume his-
tory near peak constriction could be related to specific patterns of lung mediators, indices of microvas-
cular leakage, or severity of tissue inflammation assessed pathologically. Methacholine constricted
(MC) animals served as a second control group for assessing the effects of direct smooth-muscle
contraction on indices of inflammation and volume history responses. Our results show that despite
similar baseline and postchallenge RL, HC and MC animals displayed significant constriction rever-
sal after a deep lung inflation, whereas AC animals did not. BAL concentrations of prostaglandin
D_2 (PGD_2), thromboxane B_2 (TxB_2), and leukotriene $C_4/D_4/E_4$ (slow reacting substance of anaphylaxis,
SRSA) were significantly elevated in both AC and HC animals compared with control and MC animals,
with AC and HC BAL differing only with respect to PGD_2 values (AC 2.4-fold higher). BAL total protein
in AC animals was significantly greater than in HC, MC, and control animals. Histopathology showed
significant peribronchial and interstitial cellular inflammation in AC animal specimens, whereas spec-
imens from HC animals had little or no inflammation. Differences in volume history responses ob-
served between equally constricted AC, HC, and MC animals may be due to differences in airway
and/or parenchymal microvascular leak and cellular inflammation.

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Introduction

Transient increases in airway caliber follow deep inhalation (DI) during constriction induced by isocapnic hyperpnea (1, 2), histamine (3), and methacholine (4–6) in animals and humans and account for the observed bronchodilator volume history response. Clinical observations in patients with chronic bronchitis and severe asthma have shown that a dilator response does not always follow a DI during bronchoconstriction (7, 8). These two different patterns of response suggest that the pathophysiology of induced obstruction and spontaneous obstruction are distinct, and that inflammation, which is thought to be important in the pathogenesis of both asthma and chronic bronchitis, may modulate volume history behavior. This has led to the hypothesis that blunting of the dilator volume history response during obstruction may be a useful noninvasive index of lung inflammation.

Several indirect lines of evidence support this view. First, smaller volume history responses follow a DI during induced obstruction in asthmatic subjects that in normal subjects (5, 9), and the degree of DI reversal is inversely related to hyperresponsiveness (5, 10), a putative index of inflammation. Second, the effects of a DI on airway caliber during spontaneous (i.e., noninduced) obstruction in asthmatic subjects are frequently of the opposite sign (i.e., after a DI there are transient decreases in airway caliber) (9), and worsening function is directly proportional to the severity of the spontaneous obstruction (11). Third, asthmatic subjects show less DI reversal after acute antigen-induced than methacholine-induced obstruction (8), and antigen-related late asthmatic responses, for which there is reasonable, though indirect, evidence of airway inflammation, reverse less after a DI than do early responses (12). Finally, DI bronchodilation at baseline in mild, stable asthmatic subjects relate inversely to bronchoalveolar lavage (BAL)-derived mediators and eosinophils (10).

In order to more directly assess the hypothesized relationship between inflammation and diminished dilator volume history response during bronchoconstriction, we studied guinea pigs that were challenged with either isocapnic hyperpnea or antigen and compared both indirect (i.e., BAL inflammatory mediator and total protein amounts) and direct (pulmonary airway and parenchymal histopathology) indices of pulmonary inflammation to volume history responses.

Methods

Materials

Male Hartley strain guinea pigs (weight 350 to 500 g) were purchased from Elm Hill Breeding Laboratories (Chelmsford, MA). All animals were specific pathogen free and virus antibody free; animals in each of the study groups were housed, fed, and handled in identical fashion and were studied within 1 wk of delivery to the animal facility.

The inspired gas for all hyperpnea challenges was 5% CO_2 balanced air (Medigas Corp., Malden, MA). Solutions of 1% (wt/wt) lyophilized bovine ovalbumin (Grade IV; Sigma Chemical Co., St. Louis, MO) used in the antigen challenge protocol were prepared using bacteriostatic saline. BAL was performed using warmed (37° C) sterile sa-

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line. Radioimmunoassay kits for quantifying prostaglandins D_2 and $F_{2\alpha}$ (PGD_2, $PGF_{2\alpha}$), thromboxane B_2 (TxB_2), and sulfidopeptide leukotrienes ($C_4/D_4/E_4$) were purchased from Amersham (Arlington Heights, IL). This panel of mediators was selected for study on the basis of published findings that suggested their potential roles in modulating constriction and inflammation in acute- and late-phase asthmatic responses. Total protein analysis, used as an indirect index of microvascular leakage, was performed according to the biuret test method. All assays were performed according to the manufacturer's directions on samples of cell-free BAL supernatants. Sensitivity, reproducibility, and precision of these assay techniques as applied in this laboratory have been described in a previous publication (15).

Experimental Protocol

Thirty-three guinea pigs were studied—12 hyperpnea-constricted (HC), 12 antigen-constricted (AC), and nine unconstricted controls (UC). Antigen sensitization was performed as previously described, using the inhalation method of Hutson and coworkers (14). Animals were considered to be sensitized after consecutive antigen exposures on Days 1 and 7 and were studied between Days 12 and 17.

Study animals were anesthetized using ketamine (100 mg/kg body weight) and xylazine (20 mg/kg), injected intramuscularly. Once surgical-level anesthesia was achieved, each animal was orotracheally intubated and esophageally cannulated using a technique previously described. Each animal was then placed in a whole-body plethysmograph, and transpulmonary pressure and tidal volume were measured using appropriately calibrated ± 50 cm H_2O and ± 5 cm H_2O Validyne pressure transducers (Validyne Corp., Northridge, CA). The analog tidal volume and transpulmonary pressure signals were processed digitally using a PDP 11/23 computer and software that calculated breath-to-breath lung resistance (RL) and dynamic elastance using the cross-correlation method of determination (15).

Each animal was secured in the plethysmograph and ventilated at 60 breaths/min at tidal volumes of 7 ml/kg, using a small-animal ventilator (Harvard Apparatus, Millis, MA). The system was demonstrated to be free of leaks. Baseline and postinflation recordings of RL were made. Deep inflations were performed using a 25 cm H_2O pressure source connected via a T-piece to the inspiratory side of the ventilator. By manually covering the T-piece opening during inspiration, the pressure source was functionally in line with the endotracheal tube, and an inspiratory pressure of 25 cm H_2O was transmitted to the animal's lungs. Deep inflation responses are reported as the preinflation value of resistance divided by the postinflation value. Postinflation values reported are those measured on the third tidal breath after deep inflation. This assured comparison of lung mechanics measured at isovolume, inasmuch as most animals required 2 to 3 breaths for FRC to return to baseline after deep inflation. Pressure and volume were continuously displayed on an oscilloscope, and any changes from baseline representative of a system problem were immediately detected visually and corrected. The endotracheal and esophageal cannulas were maintained free of secretions by gentle, frequent suctioning.

After completion of three to four reproducible baseline recordings, the animals were studied as follows. HC animals were challenged by exposing them to dry 5% CO_2 balanced air at increasing minute ventilations. Eight-minute hyperpnea challenges were performed, and animals were observed after challenge for bronchoconstriction for at least 10 min. Repeat challenges were performed in sequential fashion by increasing the respiratory rate at fixed tidal volume from 60 to 90 to 110 to 150 breaths per minute until at least a 100% increase in RL was observed in the posthyperpnea period. Serial resistance measurements and deep inflation responses were then recorded at 1-min intervals until a peak response in RL was noted, as indicated by a leveling off or drop in RL from maximum on serial measurements. BAL was then performed through the endotracheal tube, using 10 ml of warmed sterile saline. AC animals were studied similarly except that a 1% nebulized ovalbumin solution was administered for 1 min via the endotracheal tube as the provocative challenge. Recordings of RL and volume history responses were made at 1-min intervals until a peak response was observed, at which time lavage was performed as described previously. UC animals had serial recordings made for 30 min and were then lavaged as described earlier. MC animals were given nebulized methacholine by inhalation at a concentration of 0.01 mg/ml. Initially, 10 breaths were given. If an increase of RL of 100% or greater was not produced, the inhalations were continued until such an increase was achieved. At the completion of each study, the animals were killed by intracardiac injection of sodium pentothal. Lung gas volume was then reduced to residual volume by manually withdrawing gas through the endotracheal tube using a syringe. Fifteen milliliters of buffered 15% formalin was then injected intratracheally, and the heart and lung were dissected from the thorax en bloc and preserved in buffered 15% formalin solution. The samples were embedded in paraffin for later sectioning, hematoxylin and eosin staining, and microscopic examination. The histopathologic assessments were made (by J. J. G.) without knowledge of the experimental interventions used.

BAL fluid was processed for total protein and mediator analysis as follows. The lavage return was placed in an iced 15-ml polypropylene tube and centrifuged under nitrogen at 1,000 × *g* for 12 min at 4° C. The cell-free supernatant was divided into four aliquots, placed into 5-ml polypropylene test tubes, and stored under nitrogen at −80° C for subsequent analysis. Mediator and total protein analysis was performed in duplicate on 0.1 ml of lavage fluid. All samples were assayed within 6 wk of sampling.

Statistics

Comparisons among AC, HC, and UC animals were performed by standard analysis of variance. In cases in which results for only HC and AC animals were compared, Student's *t* test was used. Statistical significance was defined as $p < 0.05$. Linear regression correlations between BAL mediator values and RL and volume history responses were performed using Pearson's method. Statistically significant relationships were defined as those for which $p < 0.05$ when calculated from *r* values.

Results

RL/Volume History Measurements

Prechallenge RL and resistance volume history are summarized in table 1. Volume history responses are presented as the pre-deep inflation value divided by the post-deep inflation value recorded two to three breaths after the inflation as described in the METHODS section. Defined in this way, the degree of postinflation reversal of constriction is proportional to the magnitude of reported volume history ratio (i.e., < 1 = constriction, 1 = no change, and > 1 = dilation). The results demonstrate that before constrictor challenge, no significant differences existed in resistance values or in volume history ratios among UC, AC, MC, and HC groups. RL increased 7.75-fold in AC and 5.33-fold in HC animals after bronchoconstrictor challenge. These differences were not significant. In AC animals, volume history during constriction was not significantly different from prechallenge baseline. In both HC and MC animals, however, significant increases of similar magnitude in volume history response ratios from prechallenge baseline were noted during acute constriction.

BAL Mediator Levels

Lavage mediator levels for all three groups are presented in table 2. UC values represent those obtained after 30 min of ventilation and volume history maneuvers. AC, HC, and MC values represent those obtained from samples collected immediately after maximal constriction as described in the Methods section. The results demonstrate significant increases in BAL amounts and concentrations of PGD_2, $PGF_{2\alpha}$, SRSA, and TxB_2 in both AC and HC animals as compared with controls. AC levels of PGD_2 were significantly greater than HC levels but were equivalent with respect to all other eicosanoid mediators. BAL total protein con-

TABLE 1

LUNG MECHANICS

	RL ($cm\ H_2O \cdot ml/s$)	RL Volume History
Prechallenge		
Control (n = 9)	0.07 ± 0.01	0.99 ± 0.08
Antigen (n = 12)	0.12 ± 0.01	0.99 ± 0.02
Hyperpnea (n = 12)	0.09 ± 0.01	1.02 ± 0.02
Methacholine (n = 5)	0.12 ± 0.01	1.06 ± 0.05
ANOVA	p = NS	p = NS
Postchallenge		
Antigen (AC)	0.93 ± 0.20	1.18 ± 0.07
Hyperpnea (HC)	0.48 ± 0.07	1.65 ± 0.12
Methacholine (MC)	0.42 ± 0.17	1.63 ± 0.26
ANOVA	$p < 0.05$*	$p < 0.05$†

* Values significantly greater than control.
† HC and MC significantly greater than AC.

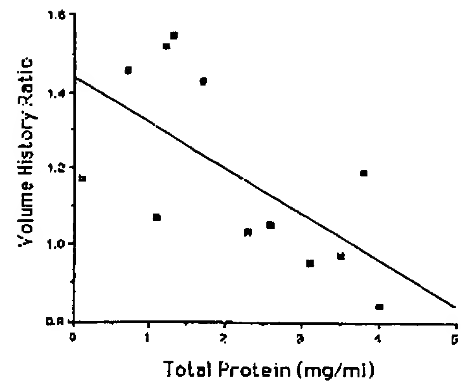

Fig 1. The volume history ratio, expressed as RL before divided by RL after a deep inflation, is shown in relation to total protein values in BAL in the antigen-constricted animals. The correlation coefficient is 0.64 with a $p < 0.025$.

centration was significantly greater in AC animals than in MC, HC, or UC animals, among which values were equivalent.

Lavage return was significantly less in AC, HC, and MC animals than in UC animals, whereas constrictor responses among AC, HC, and MC animals were equivalent. Inasmuch as methacholine is a direct smooth-muscle constrictor agonist, it should produce little, if any, direct eicosanoid mediator release. However, nonspecific release of mediators resulting from increased tissue shear stress or vagal stimulation could occur in response to methacholine administration. Our results argue against such a process, however. Despite constrictor responses and BAL returns similar to those seen in both the AC and HC test groups, mediator values in the methacholine-constricted animals were not significantly different from those among the UC animals, and were significantly less than those observed in either AC or HC animals.

Correlation among Resistance, Volume History Ratios, and BAL Mediator and Total Protein Concentrations

We observed statistically significant positive linear correlations between RL and BAL concentrations of PGD_2 ($r = 0.63$), SRSA ($r = 0.59$), and TxB_2 ($r = 0.66$). These findings are similar to those reported in AC and HC animals in previously published separate studies (13, 16). We also observed significant inverse linear correlations between BAL concentrations of both PGD_2 and total protein, and volume history ratios among AC animals (figure 1). There was no such correlation in HC animals.

Histopathology of Lung Tissue from AC and HC Animals

Light microscopic histopathologic examination was performed on lung tissue from six randomly selected animals—three from the AC and three from the HC group. All three samples from the AC animals had evidence of granulocytic and lymphocytic infiltration in the bronchial walls. This was severe in two and moderate in the remaining one. By contrast, the tissue from the HC animals had no inflammation in two and only mild granulocytic infiltration in one. Photomicrographs of representative samples are presented in figure 2.

Discussion

The results of the present study demonstrate that there is a smaller volume history response after antigen constriction than after equivalent isocapnic hyperpnea-induced constriction in guinea pigs. Although the amounts of sulfido-peptide leukotrienes, TxB_2, and $PGF_{2\alpha}$ were similar in the HC and AC groups, BAL concentrations of total protein and PGD_2 were significantly increased in the AC group. In addition, the degree of airway and parenchymal inflammation assessed histopathologically was greater in the AC group. These results support the assertion that bronchoconstriction related to or accompanied by airway and parenchymal inflammation is associated with a diminished dilator volume history response and suggest that such a blunted response may be a useful indirect index of lung inflammation in this setting.

The potential importance of differentiating bronchoconstriction associated with, or in fact arising from, inflammation and that related to transient airway smooth-muscle constriction is well recognized inasmuch as both therapy and

TABLE 2

BRONCHOALVEOLAR LAVAGE (BAL) MEDIATOR AND TOTAL PROTEIN RESULTS AND LAVAGE RETURN*

Animals	Eicosanoid Mediators (pg/0.1 ml BAL sample)				Total Protein	BAL Return
	PGD_2	$PGF_{2\alpha}$	SRSA	TxB_2	(mg/ml)	(ml out of 10 ml)
Nonconstricted control (n = 9)	5 ± 1	7 ± 1	16 ± 1	26 ± 7	0.6 ± 0.1	7.7 ± 0.8
Antigen-constricted (AC) (n = 12)	34 ± 10	38 ± 12	67 ± 16	230 ± 25	2.4 ± 0.9	2.7 ± 0.9
Hyperpnea-constricted (HC) (n = 12)	22 ± 3	59 ± 9	33 ± 3	209 ± 14	0.8 ± 0.4	3.5 ± 0.7
Methacholine control (n = 5)	10 ± 3	6 ± 2	15 ± 5	52 ± 13	0.9 ± 0.2	3.6 ± 0.9
	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

* AC and HC animals have significantly increased levels of all BAL eicosanoid mediators compared with controls (both nonconstricted and methacholine controls). AC and HC animals differ only with respect to BAL levels of PGD_2. AC animals have significantly higher BAL total protein values than HC and both control group animals. BAL return in AC, HC, and methacholine controls was significantly reduced compared with unconstricted animals.

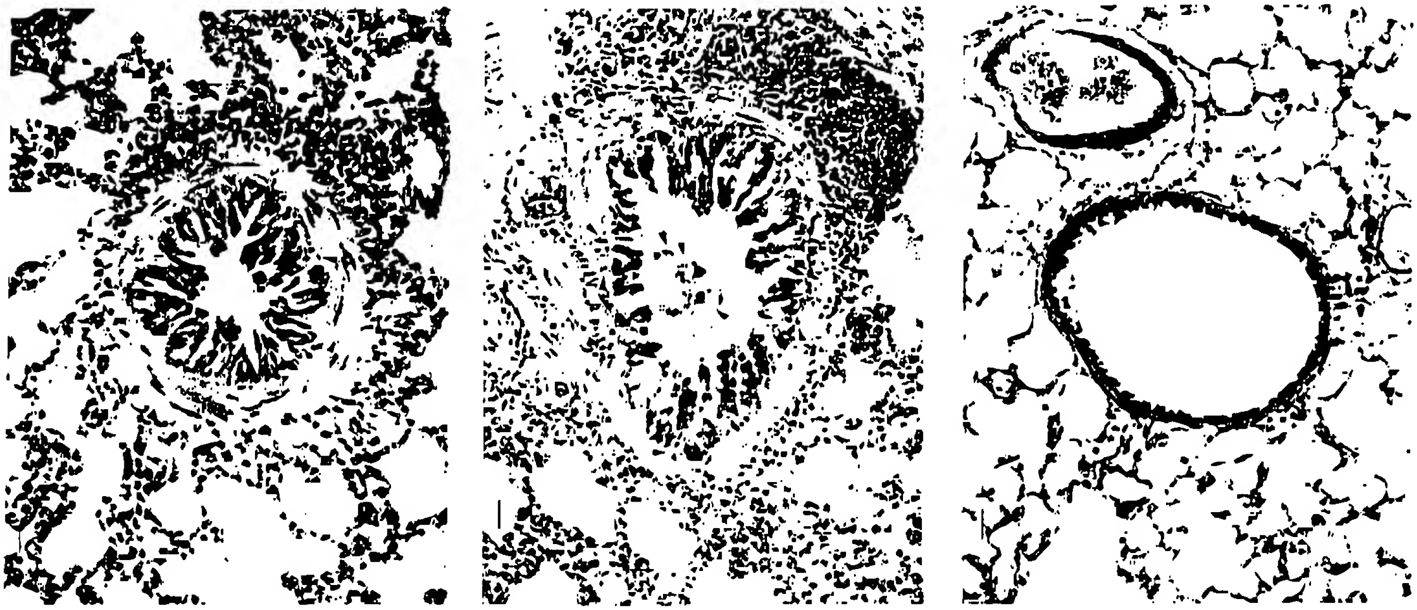

Fig. 2. (A and B) Histologic sections from antigen-constricted animals, stained with hematoxylin and eosin, showing the range of lymphocytic and granulocytic (*arrowheads*) infiltration in the airways and peribronchial tissue. Bar = 50 µm. (C) Sample from a hyperpnea-constricted animal, showing no infiltration and no evidence of edema. Bar = 50 µm.

prognosis differ. Indirect evidence from both human and animal studies supports the assertion that these represent distinct pathophysiologic states. Antigen challenges in humans, sheep, and guinea pigs have been associated with both acute- and late-phase constrictor responses as well as biochemical and histopathologic evidence of inflammation (12–14, 17). In contrast, isocapnic hyperpnea, though associated with acute constriction and mediator release in both humans and animals (16, 18), does not produce a late-phase response or induce a hyperresponsive state (19, 20, 21) and is not thought to be associated with a significant inflammatory response. Thus, the two stimuli appear to produce obstructive physiology by different mechanisms, one involving inflammation.

Previously published data from Ray and associates (22) indicate that the response to hyperpnea in the guinea pig is practically abolished by pretreatment with capsaicin, indicating that tachykinins play a pivotal role. By contrast, capsaicin pretreatment did not affect either the mechanical response or the mediator release in the sensitized guinea pig challenged with antigen (15), suggesting that differences in the underlying cellular and biochemical pathogenesis of the two states exist, even though some similarities in BAL and serum mediator profiles have been reported.

The data presented here provide direct evidence that a diminished volume history response does relate to the histopathologically demonstrable inflammation that was seen only in the antigen-challenged animals. Interestingly, in these animals the volume history ratio related inversely to the magnitude of the microvascular leak (figure 2A) and PGD_2 values (figure 2B), suggesting a spectrum of volume history responses that relates directly to these indices of severity of inflammation. Because both the antigen- and hyperpnea-challenged groups had equivalent increases in sulfidopeptide leukotrienes, TxB_2, and $PGF_{2\alpha}$, it is unlikely that these mediators account for the cellular infiltrate present or the differences in volume history ratios. The greater elevations of total protein and PGD_2 in the BAL of antigen-challenged animals suggest these indices are better predictors of cellular inflammatory infiltration and altered volume history response during constriction. However, we would not propose a direct functional role for either of these factors on the basis of the experiments presented. Rather, we view the BAL differences as indicators that antigen and hyperpnea challenges, despite causing equivalent obstruction, are different and that the histopathologic and volume history ratio differences may relate mechanistically.

Thus, although the present study does not provide any direct insight into the mechanisms acc unting for diminished volume history responses associated with inflammation, it argues that the volume history response represents a physiologic and noninvasive index of airway and parenchymal inflammation in an animal model of the asthmatic lung.

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References

1. Pichurko BM, Ingram RH Jr. Effects of airway tone and volume history on maximal expiratory flow in asthma. J Appl Physiol 1987; 62:1133–40.
2. O'Cain GF, Dowling NB, Slutsky AS, *et al.* Airway effects of respiratory heat loss in normal subjects. J Appl Physiol 1980; 49:875–80.
3. Brown NE, McFadden ER Jr, Ingram RH Jr. Airway responses to inhaled histamine in asymptomatic smokers and non-smokers. J Appl Physiol 1977; 42:508–13.
4. Burns CB, Taylor WR, Ingram RH Jr. Effects of deep inhalation in asthma: relative airway and parenchymal hysteresis. J Appl Physiol 1985; 59:1590–6.
5. Kariya ST, Thompson LM, Ingenito EP, Ingram RH Jr. Effect of lung volume, volume history and methacholine on tissue variance in man. J Appl Physiol 1989; 66:977–82.
6. Ludwig M, Dreshaj I, Solway J, Munoz A, Ingram RH. Partitioning of pulmonary resistance during constriction in the dog: effects of volume history. J Appl Physiol 1987; 62:807–15.
7. Lim TK, Ang SM, Rossing TH, Ingenito EP, Ingram RH Jr. The effects of deep inhalations on maximal expiratory flows during intensive treatment of spontaneous asthmatic episodes. Am Rev Respir Dis 1987; 140:340–3.
8. Fish JE, Ankin MG, Kelly JF, Peterman VI. Regulation of bronchomotor tone by lung infla-

tion in asthmatic and nonasthmatic subjects. J Appl Physiol 1981; 50:1079-86.
9. Sestier M, Cartier A, Martin R, Malo J. Bronchial responsiveness to methacholine and effects of respiratory maneuvers. J Appl Physiol 1984; 56:122-8.
10. Pliss LB, Ingenito EP, Ingram RH Jr. Responsiveness, inflammation, and effects of deep breaths on obstruction in mild asthma. J Appl Physiol 1989; 66:2298-304.
11. Lim TK, Pride NB, Ingram RH Jr. Effects of volume history during spontaneous and acutely induced obstruction in asthma. Am Rev Respir Dis 1987; 135:591-6.
12. Pellegrino R, Violante B, Crim E, Brusasco V. Effects of deep inhalation during early and late asthmatic reactions to allergen. Am Rev Respir Dis 1990; 142:822-5.
13. Ingenito EP, Pliss LB, Martins MA, Ingram RH Jr. Effects of capsaicin on mechanical, cellular, and mediator responses to antigen in sensitized guinea pigs. Am Rev Respir Dis 1991; 143:572-7.
14. Hutson PA, Church MK, Clay TPO, Miller P, Holgate ST. Early and late phase bronchoconstriction after allergen challenge of nonanesthetized guinea pigs. Am Rev Respir Dis 1988; 137:548-57.
15. Buxco Electronics Inc. Pulmonary mechanics, methods of computation. Sharem, CT: Buxco, 1977.
16. Ingenito EP, Pliss LB, Ingram RH, Pichurko BM. Bronchoalveolar lavage cell and mediator responses to hyperpnea-induced bronchoconstriction in the guinea pig. Am Rev Respir Dis 1990; 141:1162-6.
17. Dworski R, Sheller JR, Wickersham WE, *et al.* Allergen-stimulated release of mediators into sheep bronchoalveolar lavage fluid. Am Rev Respir Dis 1989; 139:46-51.
18. Pliss LB, Ingenito EP, Ingram RH Jr, Pichurko BM. Assessment of bronchoalveolar cell and mediator response to isocapnic hyperpnea in asthma. Am Rev Respir Dis 1990; 142:73-8.
19. Zawadski DK, Lenner KA, McFadden ER Jr. Effect of exercise on nonspecific airway reactivity. J Appl Physiol 1988; 64:812-6.
20. Rubinstein I, Levinson H, Slutsky AS, *et al.* Immediate and delayed bronchoconstriction after exercise in patients with asthma. N Engl J Med 1987; 317:482-5.
21. Zawadski DA, Lenner KA, McFadden ER Jr. Re-examination of the late asthmatic response to exercise. Am Rev Respir Dis 1988; 137:837-41.
22. Ray DW, Hernandez C, Leff FAR, Solway J. Tachykinins mediate bronchoconstriction elicited by isocapnic hyperpnea in guinea pigs. J Appl Physiol 1989; 66:1108-12.